

## Cytosolic Metal Speciation Studies by Sequential, On-line, SE-IE/HPLC-ICP-MS

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### ABSTRACT

*A procedure involving directly coupled HPLC-ICP-MS is described for the quantification of metals associated with cytosolic proteins. Selectivity is achieved by sequential fractionation by size exclusion (TSK SW2000) followed by ion exchange chromatography (Showdex DEAE). Spectra on up to 11 masses for six elements were acquired simultaneously by scanning the quadrupole in the peak hopping mode. A flow injection loop inserted downstream of the columns was used to monitor analyte recovery and quantify the ion intensity profiles from the MS. Reproducibility of analysis was approximately 2-5% and absolute detection limits were typically between 10 and 60 pg of analyte. The utility of the technique for: (i) detecting abnormal distributions in cytosolic metals due to metal exposure; and (ii) determining the biological turnover of Cu and other metals associated with proteins is demonstrated using the marine shellfish *Littorina littorea* exposed to elevated concentrations of Cd and  $^{65}\text{Cu}$ .*

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### INTRODUCTION

It is generally assumed that most metals exert their toxicity by non-specifically binding and inactivating sensitive target macromolecules critical for normal cellular functioning (Mason and Jenkins, 1995). Progress in identifying the molecular targets susceptible to inactivation and the cellular moieties involved in restricting the extent of non-specific binding has been limited because the analyses require not only the separation and characterization of the various metal binding species, but also qualitative and quantitative data on the various associated metals.

The purpose of the present study was to assess the potential of a new technique involving the sequential coupling of size exclusion (SE) and an ion exchange (IE) HPLC system to an inductively coupled plasma mass spectrometer (ICP-MS) for studying metallo-protein speciation and identifying imbalances in metal homeostasis. This procedure

chromatographically fractionates samples based upon size and charge while simultaneously identifying and quantifying the isotopic abundance of associated metals. In addition to being highly selective and sensitive, the technique has the added virtue that, because elemental identification is based upon mass, it can monitor the long-term movements of certain metabolically important metals, such as Cu, which have multiple stable isotopic forms and can not be studied conventionally because of a lack of convenient radio tracer.

The capabilities of this new technique are illustrated by examining the cytosolic distribution of Cd and Cu and other metals within the visceral complex of the marine mollusc *Littorina littorea*.

## MATERIALS AND METHODS

### Exposure regime

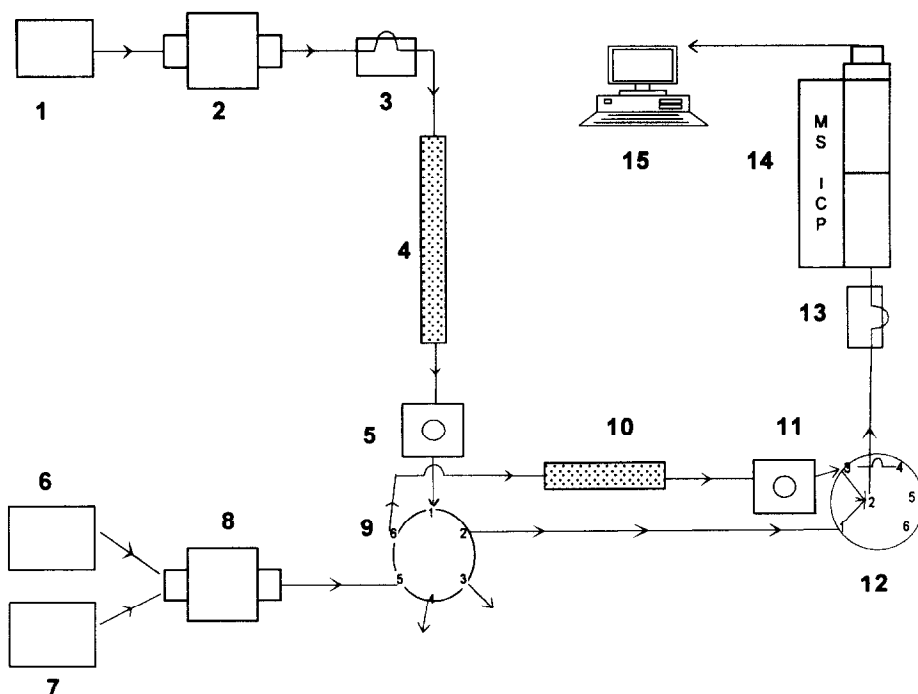
Two groups of 10 Littorinids were exposed to seawater containing pure, stable isotopic  $^{65}\text{Cu}$  ( $10^{-11}\text{ M}$ ) and either low ( $10^{-11}\text{ M}$ ) or elevated concentrations of Cd ( $10^{-8}\text{ M}$ ). The free ion activities of  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  were controlled using a NTA-based metal-chelate buffer system and the medium was replaced weekly.

### Sample preparation

The two groups of animals were removed after 42 d exposure and the visceral complexes were dissected upon ice, pooled, weighed and homogenized at  $4^{\circ}\text{C}$  in 3 vol (w/v) of ice-cold, 200 mM Tris buffer containing 2 mM dithiothreitol (DTT) in 0.1% sodium azide (pH 8.5). Phenylmethanesulfonyl fluoride was added to the sample at a final concentration of 0.1 mM immediately prior to homogenization. The homogenates were centrifuged at 100 000 g for 1 h to produce a cytosolic fraction for analysis by HPLC-ICP-MS.

### Size exclusion-ion exchange-high performance liquid chromatography-inductively coupled plasma mass spectroscopy (SE-IE/HPLC-ICP-MS)

A schematic diagram of the SE-IE/HPLC-ICP-MS is shown in Fig. 1. Aliquots ( $100\text{ }\mu\text{l}$ ) of cytosol were fractionated at a flow rate of  $1\text{ ml min}^{-1}$ , either isocratically by size-exclusion (SW 2000 TSK Column) using a mobile phase of 20 mM Tris (pH 7.2) containing 1 mM DTT or sequentially by size-exclusion followed by ion-exchange HPLC. A switching valve (#9, Fig. 1) was used to re-direct eluting species of interest from the size exclusion to the ion-exchange (Showdex DEAE-825) column. A linear gradient of 20 mM Tris (pH 8.2) containing 1 mM DTT increasing to 250 mM Tris in 500 mM  $\text{NH}_4\text{Cl}$  and 1 mM DTT over 60 min was used to fractionate the proteins from the IE column. The UV absorbance of the eluent was monitored at either 254 or 280 nm by two detectors positioned downstream of the columns. A  $20\text{-}\mu\text{l}$  flow injection loop installed immediately prior to the ICP-MS was used to quantify the elemental composition of the resolved peaks and monitor analyte recovery (Mason *et al.*, 1990). A VG Plasma Quad  $2^{+}$ , operated in the time-resolved mode, was used to simultaneously acquire data on up to six elements. Chromatographic analysis was performed using MassLynx integration software.

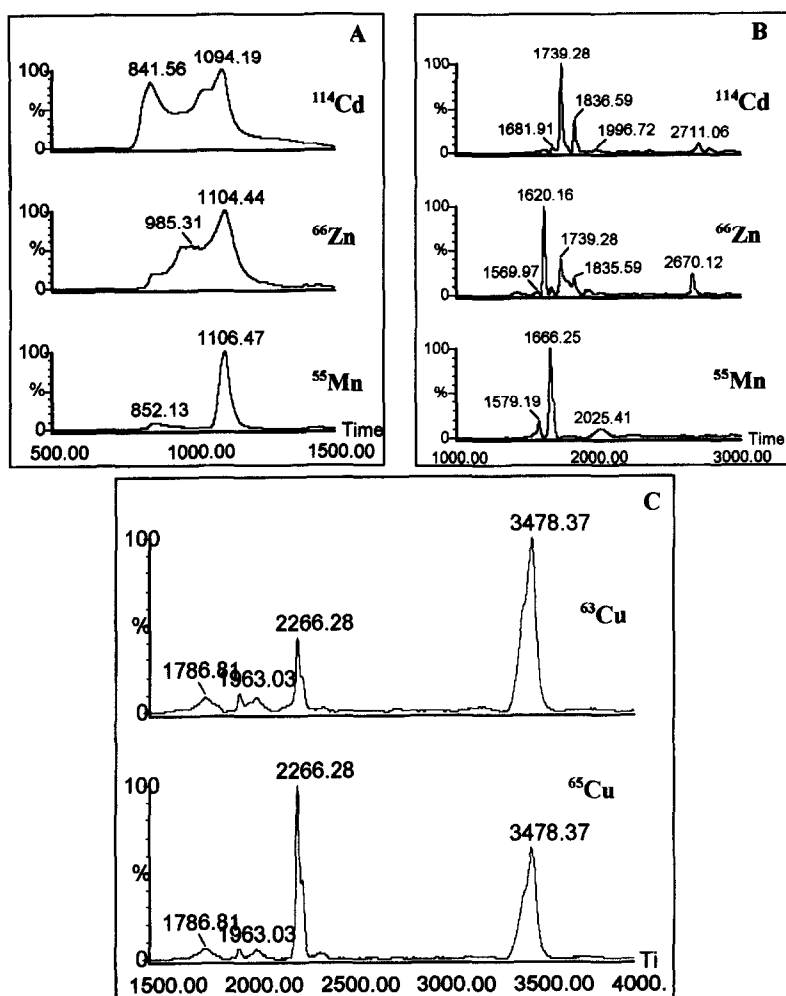


**Fig. 1.** Schematic diagram showing the configuration of the SE-IE/HPLC-ICP-MS used in this study. The number-labels correspond to the following instrument components: (1) mobile phase composed of 20 mM Tris and 1 mM DTT (pH 7.2); (2) HPLC pump A; (3) Rheodyne 7125, 100- $\mu$ l injection loop; (4) TSK SW2000 size exclusion column; (5) 254/280 UV detector; (6, 7) mobile phase for gradient ion exchange: 20 mM Tris in 1 mM DTT, pH 8.2; 250 mM Tris, 500 mM  $\text{NH}_4\text{Cl}$  in 1 mM DTT, pH 8.2; (8) HPLC pump B; (9) Rheodyne 7000 switching valve; (10) Shodex DEAE-825 ion exchange column; (11) 254/280 UV. detector; (12) Rheodyne 7000 switching valve; (13) Rheodyne 7125 20- $\mu$ l injection loop; (14) VG Plasma Quad 2 + ICP-MS; (15) computer terminal-time resolved analysis and VG MassLynx software.

## RESULTS AND DISCUSSION

Most techniques developed to study metal speciation in cells utilize a two-step procedure. The initial step normally involves some form of separatory technique to isolate the various metallo-complexes, either temporally or spatially. During step two, the fractionated components are analysed for metal content. Ideally, the former should provide a high degree of selectivity, while the latter should exhibit good sensitivity and detection limits. One of the most successful combinations in this respect because of its sensitivity, speed of analysis and multi-elemental capability, has been the use of ICP-MS as an elemental detector for HPLC. For practical reasons, cytosolic matrices have typically been fractionated isocratically by size exclusion chromatography prior to ICP-MS analysis (Mason *et al.*, 1990; Owen *et al.*, 1992; Mason and Storms, 1993; High *et al.*, 1995; Suzuki *et al.*, 1995). While this form of chromatography tends to retain the native configuration of the molecule and has enabled the relative masses of metallocomplexes to be determined, the procedure suffers from a lack of selectivity. Consequently, elemental profiles commonly show

overlapping peaks caused by the co-migration of unresolved species (Mason *et al.*, 1990). Since co-elution of essential and non-essential metals is commonly used as evidence for metal toxicity via competitive substitution, this lack of resolution can compromise the utility of the technique for toxicological studies. Some of the difficulties associated with the interpretation of mass spectra obtained by SE-HPLC/ICP-MS are illustrated in Fig. 2(A). For example, the unresolved peak eluting between 1094 and 1106 s having an apparent



**Fig. 2.** (A) SE-HPLC/ICP-MS profiles for  $^{114}\text{Cd}$ ,  $^{66}\text{Zn}$  and  $^{55}\text{Mn}$ , obtained from the visceral complex cytosol of snails exposed to  $10^{-8}$   $[\text{Cd}^{2+}]$  for 8 weeks. The isotope abundances are expressed as intensity units relative to a maximum response for each respective isotope (% intensity). (B) IE-HPLC/ICP-MS profile showing further fractionation of the unresolved moieties in (A). (C) IE-HPLC/ICP-MS profile of a cytosol from a  $^{65}\text{Cu}$ -exposed animal. Comparisons of the  $^{65}\text{Cu}$  and  $^{63}\text{Cu}$  profiles illustrate that the protein eluting at 2266 s is either accumulating or turning over Cu faster than the protein eluting at 3478 s. The natural isotopic abundance ratio of  $^{65}\text{Cu}$ :  $^{63}\text{Cu}$  under the current operational conditions was 2.1.

molecular weight of approximately 18 kD appears to bind Cd, Zn and Mn, implying that this might represent a site of toxicological impact for Cd. However, elemental profiles from a duplicate sample fractionated first by size and then charge (Fig. 2(B)) show the presence of Mn- (1579 and 1666 s), Zn- (1620 and 2670 s) and Cd-specific species (2711 and 2820 s). Co-elution of Cd with Zn can be observed at 1739 and 1836 s which probably corresponds to the two molluscan isoforms of metallothionein (Berger *et al.*, 1995).

One of the additional advantages of using an MS detector is that it permits the use of enriched stable isotopes as tracers in metabolic studies. For elements, such as Cu, which lack a convenient radioisotope, this approach represents the only methodology for studying the long-term turnover of the metal in proteins. The application of this procedure is illustrated in Fig. 2(C) which shows the relative abundances of  $^{63}\text{Cu}$  and  $^{65}\text{Cu}$  in two peaks eluting at 2266 and 3478 s from a SE-IC/HPLC profile obtained from the visceral complex cytosol of a  $^{65}\text{Cu}$  exposed animal. Calculations, based on the response from a 20 ng FIA injection of Cu standard indicate that the former and latter peaks contained 2.04 ng  $^{63}\text{Cu}$  and 5.15 ng  $^{65}\text{Cu}$ , and 18.90 ng  $^{63}\text{Cu}$  and 10.79 ng  $^{65}\text{Cu}$ , respectively. Accumulation of  $^{65}\text{Cu}$  from the media, therefore, accounted for 58.5 and 6% of the total Cu content of these proteins, demonstrating either rapid turnover or accumulation of Cu by the former. The identity of these peaks and the biological significance of these findings remains to be determined.

In conclusion, the major virtue of coupling SE and IC chromatographic systems in tandem before ICP-MS is an increase in selectivity. Although the required use of high ionic strength buffers tends to cause ion suppression (7, 8, 9, 10 and 13% for  $^{63}\text{Cu}$ ,  $^{65}\text{Cu}$ ,  $^{66}\text{Zn}$ ,  $^{55}\text{Mn}$  and  $^{114}\text{Cd}$ , respectively) and increase background signal, the absolute detection limits (3 $\sigma$ ) are still superior to most other forms of analysis (12, 37, 60, 8 and 18 pg for  $^{63}\text{Cu}$ ,  $^{65}\text{Cu}$ ,  $^{66}\text{Zn}$ ,  $^{55}\text{Mn}$  and  $^{114}\text{Cd}$ , respectively).

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